Role of Lipids in the pathogenesis of Dengue
- A prospective cohort study

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ABSTRACT

Background

Dengue virus causes illnesses with or without warning indicators for severe complications. Sphingolipids are crucial for vascular integrity and cellular homeostasis, with recent studies highlighting their role in viral diseases.Still, there are no clear prognostic signs linked to the disease outcomes.

Objectives

The study aimed to assess the plasma levels of sphingolipids, specifically Sphingosine -1-phosphate (S1P) and the key enzymes of sphingolipid metabolism: Sphingomyelin synthase (SGMS1), Ceramide Kinase (CERK) and acid ceramidase (ASAH1) and its association with clinical outcomes of dengue.

Methods

This prospective cohort study had 102 dengue cases with 17 severe dengue (SD), 33 dengue with warning signs (DWW), 52 dengue without warning signs (DWOW) along with 10 each from other febrile illnesses and healthy controls. Blood was collected across febrile, defervescence, and convalescence phases. Dengue was confirmed by serological and molecular assays. Clinical and blood parameters were documented during the febrile and critical phases and analyzed statistically. Plasma levels of S1P and the enzymes were measured using ELISA, mRNA using qRT-PCR. Predictive efficacy was determined using Support vector machine (SVM) models.

Results

Molecular analysis revealed that DENV-2 and DENV-3 stood out as the primary serotype, frequently presenting with severe symptoms. The study results found that clinical signs such as bleeding and nausea, platelet counts, and USG were linked with disease severity. SVM analysis revealed that the IgG/IgM along with other clinical symptoms including USG findings, could predict the disease severity with maximum accuracy. The study showed a significant reduction in S1P levels across all dengue forms during febrile phases, with further decline in SD during the critical phase ($P < 0.05$). mRNA levels of the enzymes were increasing during critical phase ($P \leq 0.001$) with no significant difference noted in their respective protein levels. S1P and SGMS1

levels correlated significantly with clinical severity indicators, including hematocrit, albumin, platelet count, and liver enzymes. SVM analysis identified CERK levels along with platelet count, HCT, and ALT as markers with high predictive accuracy for dengue severity.

Conclusion

Overall, USG testing at the earlier phase of dengue infections will be greatly helpful for the early prediction of severe dengue. The study reports an association of sphingolipids with dengue virulence, emphasizing the role of S1P metabolism in disease progression and plasma leakage, and highlighting the potential of targeting sphingolipids in managing severe dengue.

Keywords: *Dengue; Support Vector Machine; Plasma Leakage; Thrombocytopenia; Sphingolipids; Serotypes; Real-time Polymerase chain reaction (RT-PCR)*

1. INTRODUCTION

Dengue viruses primarily affect tropical regions, with approximately 3.9 billion people worldwide at risk(1).These viruses are transmitted to humans through Aedes mosquitoes, predominantly *Aedes aegypti,* followed by *Aedes albopictus.* A primary dengue virus infection usually causes mild fever, while severe forms of the disease, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), can be life-threatening(2).The dengue virus belongs to the Flaviviridae family and has four closely related serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. These serotypes can lead to various clinical symptoms, and the sequence or timing of infections may influence disease severity and prognosis. Dengue fever is classified as mild, moderate, or severe, depending on the patient's immune response to the infection(3).

Recovery from a primary infection by one serotype provides lifelong protection against that particular serotype(4). However, cross-immunity to other serotypes is only partial after recovery(5). In addition, subsequent infections by other serotypes, termed secondary infections, increase the risk of developing severe dengue(6). Aside from the fact that severity is related to secondary dengue infection, a recent study has reported that severity occurred in both primary and secondary dengue in pediatric cases(7). A rise in dengue cases has been observed from 2015 to 2024, with a sharp surge in 2024. According to WHO Global Dengue Surveillance, 2019 saw a significant spike to 3.5 million cases, followed by a slight decline in 2020 and 2021. The numbers then escalated again in 2022 and 2023, reaching 5.27 million cases. By August 2024, dengue cases had soared to an alarming 12.06 million,

marking a severe outbreak. The death toll also rose sharply, with 6,991 fatalities recorded(8).

As of February 21, 2024, India recorded 1,86,567 dengue cases and 160 deaths, according to the National Vector Borne Disease Control Program (NVBDCP)(9). Among these, Tamil Nadu reported 19,138 cases with 7 deaths, while Puducherry had 3,689 cases with no mortality. Currently, there are no specific treatments or vaccines available for dengue. WHO classifies the clinical spectrum of dengue infection into three categories: dengue without warning signs (DWOW), dengue with warning symptoms (DWWS), and severe dengue (SD). The initial phase (DWOW) resembles flu-like symptoms such as fever, headache, nausea, vomiting, abdominal discomfort, joint and muscle pain, and rash(5).

The critical phase of dengue begins with warning signs, particularly following defervescence. These signs include abdominal pain, recurrent vomiting, fluid accumulation, mucous membrane bleeding, fatigue, restlessness, an enlarged liver, and a rise in hematocrit (HCT) values(10).Continuous monitoring of patients with fluid therapy is needed for proper patient management(11). About 2 to 3% of dengue patients progress to SD, which includes DHF and DSS. DHF is caused by severe thrombocytopenia and bleeding, and the most critical phase includes severe organ impairment, shock, and substantial plasma leakage, leading to dengue shock syndrome (DSS).The main causes of death from dengue fever include hypovolemic shock, severe bleeding, respiratory failure, and other organ involvement(12).The DENV virus primarily targets plasma-released lipids, monocytes/macrophages, endothelial cells (ECs), and platelets, resulting in thrombocytopenia and endothelial dysfunction. These two crucial cellular events contribute to disease pathogenesis.

Currently there is no specific therapeutics or effective-monitoring methods are available for the management of dengue. Identifying prognostic markers for the severity of dengue fever is essential to reducing mortality associated with severe manifestations and complications.

Sphingolipids, a class of lipids that plays a major role in cellular homeostasis are recently reported to have pivotal role in regulating vascular integrity. Though many lipids are reported to be altered during dengue viral infection, the role of sphingolipids in the pathogenesis of dengue is not explored. To address this gap, the present study was designed to understand the plasma levels of selected sphingolipids and their key enzyme molecules CERK, ASHA1 and SMS1 in the pathogenesis of dengue.

2. AIMS AND OBJECTIVES

2.1 Aim: To study the levels of plasma sphingolipids and their key enzymes presumed to be involved in vascular dysfunction and disease pathogenesis of severe dengue.

2.2 Primary objectives

1. To assess the Clinical and biochemical profile of dengue cases for the detection of severity markers using machine learning approach.

2. To quantify selected transcripts of sphingolipid markers in severe and non-severe dengue compared to other febrile illnesses using qRT-PCR.

3. To estimate the expression levels of selected sphingolipid markers in severe and non-severe dengue compared to other febrile illnesses using ELISA.

2.3 Secondary objectives

4. Quantification of selected lipids by HPLC and to validate its efficacy in predicting severe form of dengue from non-severe forms.

3. HYPOTHESIS

Levels of plasma sphingolipid and the key enzymes involved in sphingolipid metabolism in severe and non-severe dengue could serve as effective markers for the early prediction of disease outcomes

4. REVIEW OF LITERATURE

4.1. Dengue classification and its clinical stages

Dengue, an arboviral infection transmitted by *Aedes aegypti*, is hyperendemic in tropical and subtropical regions. Its rising incidence puts nearly half the global population at risk. Its clinical spectrum varies from asymptomatic to severe multi-organ failure(13).The illness progresses through febrile, critical, and recovery phases, with plasma leakage marking DHF soon after the fever subsides. This leakage is transient and dynamic, lasting 24-48 hours. Developing a dengue vaccine is complex due to four serotypes, though tetravalent candidates are in progress.

4.2 Role of lipids in Cellular Function and Immune Regulation

Lipids are a diverse group of biomolecules essential for cellular structure, energy storage, and signaling(14).The lipid bilayer of cell membranes maintains cellular integrity and facilitates signal transduction. Beyond their structural roles, lipids act as signaling molecules, including phospholipids, sphingolipids, and eicosanoids, which regulate inflammation, immune responses, and apoptosis(15). Lipids significantly influence both innate and adaptive immunity, affecting the host's inflammatory response, endothelial integrity, and vascular permeability during viral infections like dengue(16). Dysregulation of lipid metabolism and lipid mediator signaling may contribute to severe dengue pathogenesis(17).Several studies have explored the intricate relationship between virus infections and sphingolipid metabolism, revealing how viruses hijack host lipid pathways for replication and immune evasion. Sphingolipids, key components of cell membranes, play crucial roles in viral entry, replication, and pathogenesis. Notably, sphingolipid-targeting therapies show promise in antiviral strategies. Studies on influenza, HIV, and SARS-CoV-2 underscore the significance of these lipids in infection dynamics, suggesting

novel therapeutic targets to disrupt viral life cycles by modulating sphingolipid metabolism(18).Altered cholesterol homeostasis influences viral replication in various cell types, including endothelial cells, monocytes, and macrophages. The expression of lipid metabolism-related enzymes, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), can be modulated by DENV(19,20). These enzymes control fatty acid synthesis, crucial for viral replication.

4.3 Sphingolipid Metabolism in Viral Infections

Cellular sphingolipids are essential for the replication and spread of human viruses, as they are actively utilized at multiple stages of the viral life cycle, including cell entry, membrane fusion, genome replication, and propagation(21).The biosynthesis of intracellular sphingolipids can induce conformational alterations in viral receptors and facilitate the escape of viral particles from endosomes. However, despite these insights, the precise molecular mechanisms by which sphingolipids regulate viral replication remain incompletely understood(22).Further research is necessary to establish a more comprehensive understanding of the interplay between sphingolipid metabolism and viral infections. Sphingolipids play a crucial role in cellular signaling and metabolism, with their metabolites essential for immune defense against intracellular pathogens. Their involvement in immune responses makes sphingolipid metabolism a promising target for vaccine and antiviral development(23).A study reported that ceramide, a key bioactive lipid, regulates membrane properties and forms specialized micro-domains. It interacts with enzymes like sphingomyelinases to influence viral infection, serving as receptors, clustering entry receptors, aiding endosomal escape, facilitating replication, and promoting the release of new virions. Recent evidence suggests that targeting enzymes involved in sphingolipid metabolism could be a promising strategy for controlling viral infections. Another study by Dai et al 2024 showed that variations in serum

sphingolipid levels could serve as potential biomarkers to monitor the progression of viral diseases in patients(24).

4.4 Lipidomic Analysis in Dengue Pathophysiology

Lipidomic analysis has emerged as a valuable tool for understanding dengue pathophysiology. Profiling lipid species in serum provides insight into metabolic alterations associated with severe dengue(25).A study reported that total of 423 metabolites were identified, with 14 showing significant differences across dengue groups. Four metabolites differed notably from healthy controls. ROC analysis highlighted 11 potential biomarkers, while logistic regression identified three key metabolites for predicting severe dengue (AUC: 0.75). Dengue impacts lipid metabolism, oxidative stress, and inflammation(26).Previous study reported that dengue virus (DENV) infection alters metabolism, relying on host cell machinery for replication. In THP-1-derived macrophages, 202 metabolic changes were observed, with amino acids, fatty acids, and carbohydrates significantly affected. DENV-induced anabolism supports viral replication and immune activation. Notably, 11 metabolites differed between standard infection and antibody-dependent enhancement(27).However, despite these insights, the precise molecular mechanisms by which sphingolipids regulate viral replication remain incompletely understood. Further research is necessary to establish a more comprehensive understanding of the interplay between sphingolipid and their key enzymes-CERK, ASHA1 and SMS1 forms a regulatory network influencing immune response, endothelial function, and viral replication in dengue infection. Based on this in the present study, the alterations in the levels of plasma lipids in dengue cases during the course of infection compared to other febrile illness and to validate the efficacy of specific potential lipids in the early prediction of severe dengue.

5. MATERIALS & METHODS

5.1 Patient recruitment and sample collection

The study was approved by the Institutional Human Ethical Review Committee (ECR/451/Inst/PO/2013/RR-16). The patient presented to General Medicine at Mahatma Gandhi Medical College and Research Institute, Pondicherry with symptoms suggestive of dengue and confirmed by antigen (NS1) and an antibody test (IgM, IgG) were included in the study. Dengue-infected cases whose infection was confirmed by NS1 antigen detection by dengue early ELISA kit or dengue IgM Capture ELISA Kit, (InBios International, Inc. USA). To classify the patients into primary and secondary infection dengue IgG capture ELISA kit was used (InBios International, Inc. USA). A DENV infection has been defined as primary if the capture IgM/IgG ratio is greater than 1.2, or as secondary if the ratio is less than 1.2. This prospective cohort study involved 102 laboratory-confirmed DENV patients, consisting of dengue without warning signs (DWOW-52), dengue with warning signs (DWW-33), and severe dengue cases (SD-17). Additionally, the study involved 10 other febrile illnesses (OFI) and 10 healthy controls (HC). Blood samples were collected at the time of admission (DOA), and the day of defervescence (DOD) and day of convalescence (DOC).

5.2 Inclusion and Exclusion criteria

Adults (18-60yrs) and who satisfy the following criteria will be included in the respective groups.

a) Subjects who are all positive for dengue NS1 antigen or anti IgM dengue antibodies (Inbios International, Seattle, USA) or virus by PCR.

b) Subjects who are with other febrile illness (OFI). OFIs is defined as those patients with short febrile illness (<2 wks) who are negative for dengue NS1 antigen, anti-IgM dengue antibodies, and no obvious bacterial rickettsia.

c) Group 3 includes subjects who are healthy volunteers.

Subjects excluded from the study are patients with a known history of smoking, chronic infection, mixed or co-infection, cancer, steroid therapy, chemotherapy& radiotherapy since the above condition can affect the level of study parameters.

5.3 Classification of samples

Patients were classified into severe dengue (SD), dengue with warning signs (DWW), and non-severe dengue without warning signs (DWOW) as per WHO 2009 criteria(28).In the present study patients classified as dengue with or without warning signs, were considered as a single group, non-severe dengue (NSD). DWOW was classified who presented a fever with any two of the following criteria: nausea, vomiting, rash, aches and pains, and leukopenia. DWW included any of the following clinical conditions: abdominal pain, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy, restlessness, liver enlargement >2 cm, and an increase in hematocrit (HCT) concentration with a rapid decrease in platelet count. SD was classified as severe plasma leakage leading to shock or fluid accumulation with respiratory distress, severe bleeding evaluated by the clinician, and severe organ involvement such as Liver (AST or ALT ≥1000), CNS (impaired consciousness), failure of the heart, and other organs.

5.4 Sample collection and processing

About 3 ml of blood sample was collected from each patient at the time of admission and at the time of discharge. Serum, plasma was separated as per standard

protocol and stored at -80° C. Once the desired number of samples was collected, all samples were processed together. PBMCs were separated using Hisep LSM 1077 (Himedia, Mumbai, India). Total RNA was extracted using the TRIZOL method, and c-DNA was prepared using the Prime Script RT Reagent Kit (Takara Bio Inc., Shiga, Japan). Dengue serotype-specific PCR was carried out using universal PCR and serotype-specific primers as described **(29)** and the results were analyzed.

5.5 PBMC isolation, RNA extraction, and cDNA synthesis

PBMCs were separated using HiSep LSM 1077 (Himedia, Mumbai, India), washed twice with PBS, stored in 1 mL of trizol reagent (Himedia, Mumbai, India), and stored at-80°c. Total RNA was extracted using the TRIZOL method (Himedia, Mumbai, India). The purity and concentration of RNA were assessed using a Nano Drop spectrophotometer (Thermo Scientific, Waltman, MA, USA). The complementary DNA (cDNA) was synthesized from 1 µg of RNA using high-capacity cDNA prepared using the Prime Script RT Reagent Kit (Takara Bio Inc., Shiga, Japan). The cDNAs thus obtained were tested for integrity by amplification of β-actin transcripts in a 40-cycle PCR. Standard curves were constructed using the mean cycle threshold (Ct) value.

5.6 PCR for DENV serotyping

DENV nucleic acid detection and serotyping were performed using conventional PCR followed by PCR product sequencing. The PCR amplified products were analyzed by electrophoresis on 1% agarose gel. The PCR products were purified using the QiAquick PCR Purification kit(Qiagen, Hilden, Germany) andsequenced. Finally, the sequencing results were subjected to BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis, and serotypes were identified.

5.7 Analysis of Sphingolipids and key enzyme molecules for gene expression using qPCR

The list of primers used to amplify the endogenous control and target genes is listed in Table 1. Briefly, the reaction mix included 5 µL of TB Green Premix Ex Taq II (Takara Bio Inc., Shiga, Japan), 0.2 µL of each primer at 10 µM (Bioserve Biotechnologies Pvt. Ltd, India), and 3.6 µL of molecular grade water (Himedia, Mumbai, India). All the reactions were carried out in triplicates with a standard run of initial denaturation at 95˚C for 30s followed by 40 cycles of denaturation (95˚C for 10s), and a combined annealing and extension step (60˚C for 30s) in the CXF96 qRT-PCR Detection System (Biorad Laboratories Inc., California, USA). The melting curve analysis was used to confirm the specificity of each primer set, and their respective PCR products were confirmed by agarose gel electrophoresis. The relative gene expression of selected transcripts within and between the groups was determined using $2^{-\Delta\Delta}$ct values. Ct is the cycle threshold, ΔCt is the difference in threshold cycles between target genes and housekeeping gene (β-actin), and ΔΔCt equals the amount of target genes normalized to actin and relative to the healthy control (Livak and Schmittgen, 2001)(30).

Table 1. Study primers for gene expression analysis

Gene Name	Sense Strand (5'-3')	Anti-Sense Strand (3'-5')
β-Actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG
SMS1	GAAGCCCAACTGCGAAGAATAA	AGAGTCGCCGAGGGGAATAC
ASHA1	AGTTGCGTCGCCTTAGTCCT	TGCACCTCTGTACGTTGGTC
CERK	CACCTTAGCCTCCATCACCACTG	AACATACCATCTCCGCCGACAC

SMS1- *Sphingomyelin synthase1,* ***ASHA1-*** *Acid Ceramidase,* ***CERK-*** *Ceramide kinase*

5.8 Estimation of plasma levels of selected molecules by ELISA

To assess the circulating levels of selected markers, a quantitative human-specific sandwich ELISA kit was used, as described by the manufacturer's protocol. The assay was carried out using SMS1 (Sphingomyelin synthase1), CERK (Ceramide

kinase) and S1P (Sphingosine-1-Phosphate) respectively. All the analysis and calibrations were carried out in duplicate, according to the manufacturer's protocol. The optical density (OD in nm) was determined at 450nm (SpectraMax M5, Molecular Devices, California, and USA). The concentration of selected markers was estimated from the standard curve determined by the serially diluted standards.

5.9 In-vitro assessment: HUVEC culture and treatment

Human Umbilical Vein Endothelial Cells (HUVEC) purchased from Lonza (Walkersville, USA) were maintained in the laboratory as prescribed ***(31)***. In brief, HUVECs were seeded (1.5 ×104/well) in a 6-well plate. Cells were starved in EBM2s basal medium for 24 hours at 37° C and 5% CO2 before incubation in media containing 10% human serum collected from SD, DWW, and DWOW ***(32)***.After 24 h of treatment, the HUVEC cells were harvested and re-suspended in PBS solution. Proteins levels were assessed using ELISA.

5.10 HPLC quantification of selected lipid (C18:1 Ceramide) to predict severe vs. non-severe dengue.

A lipid standard, Ceramide (d18:1/18:1(19Z)) (Avanti Polar, Alabaster, Alabama, USA), along with an internal standard, Ceramide (d18:1/17:0), was added to all test samples prior to lipid extraction to standardize raw peak areas and account for extraction variability. Plasma ceramide quantification was performed using the Alshehry Extraction Method, as previously reported. The extracted derivatives were analyzed on a Shimadzu HPLC system equipped with an RP-18 Shimadzu column (4.6 mm × 250 mm) maintained at 40°C. A 20 µL sample mixture was injected into the column, with a flow rate of 1.000 mL/min sustained for 20 minutes. The target peak's retention time was identified using an emission wavelength of 274 nm.

5.11 Support Vector Machine (SVM) learning model to predict disease severity

This study was conducted in collaboration with CSIR-IICT, Hyderabad, India. Clinical data pre-processing was carried out using the Simple Imputer function. For binary classification, various machine learning models were employed. The dataset was split into an 80-20 training-test ratio, with the training set further tuning using three-fold cross-validation (k=3). To predict dengue severity, an SVM-based binary classification approach was applied across three comparisons: DWOW vs. DWW, DWOW vs. Severe, and DWW vs. Severe. Feature importance was determined using the Select from Model technique, and bar plots were generated for visualization. Model performance was evaluated based on accuracy, Area under the Curve (AUC), and F1 Score, ensuring the models' effectiveness and reliability in classifying dengue severity levels.

5.12 Statistical analysis

Continuous variables were presented as mean ± standard deviation, while discrete variables were expressed as numbers and percentages. Mean comparisons between two study groups were assessed using the unpaired t-test, and differences in proportions were analyzed using the chi-square test or Fisher's exact test. Associations between markers and SD were evaluated through the chi-square test and univariate logistic regression. Data were presented as median with interquartile range, and normality was tested using the Shapiro-Wilk test. Within-group comparisons were performed using the two-tailed related sample t-test (Wilcoxon Signed Rank Test), while between-group comparisons used the two-tailed Independent Sample t-test (Mann-Whitney U test). Spearman rank correlation was applied to assess the relationship between sphingolipid expression and clinical characteristics. A p-value < 0.05 was considered statistically significant. Statistical analyses were conducted using SPSS 27 (SPSS, Chicago, IL, USA).

6. RESULTS

6.1 Demographic and clinical profile of study participants

Table 2. Clinical signs and Symptoms of the study group at the time of admission

Variables		DWOW (N=52)	DWW(N=33)	SD (N=17)	P-value
Age		32.23±13.81	27.70±10.14	35.29±17.19	0.131
Heart rate in min		**82.65±12.84**	**87.89±14.59**	**91.94±12.77**	**0.032**
Systolic BP		114±12.09	115.76±18.55	120±15.00	0.363
Diastolic BP		72.46±10.24	73.03±13.11	76.47±9.31	0.431
Days of fever		4.12±1.43	4.23±1.51	3.94±2.24	0.813
Length of the Hospital stay		6.72±1.42	6.44±1.41	6.875±2.47	0.565
Gender	Male	28(53.85%)	25(75.76%)	11(64.71%)	0.124
	Female	24(46.15%)	8(24.24%)	6(35.29%)	
Headache	No	31(59.62%)	13(39.39%)	7(41.18%)	0.140
	Yes	21(40.38%)	20(60.61%)	10(58.82%)	
Aches & Pains	No	28(53.85%)	15(45.45%)	5(29.41%)	0.210
	Yes	24(46.15%)	18(54.55%)	12(70.59%)	
Nausea	**No**	**42(80.77%)**	**23(69.70%)**	**7(41.18%)**	**0.008**
	Yes	**10(19.23%)**	**10(30.30%)**	**10(58.82%)**	
Vomiting	No	30(57.69%)	15(45.45%)	8(47.06%)	0.495
	Yes	22(42.31%)	18(54.55%)	9(52.94%)	
Abdominal Pain	No	41(78.85%)	21(63.64%)	9(52.94%)	0.087
	Yes	11(21.51%)	12(36.36%)	8(47.06%)	
Bleeding	**No**	**51(100%)**	**20(57.58%)**	**9(52.94%)**	**0.001**
	Yes	**1(0%)**	**13(42.42%)**	**8(47.06%)**	
Dry cough	No	44(84.62%)	23(69.70%)	14(82.35%)	0.240
	Yes	8(15.38%)	10(30.30%)	3(17.65%)	
Rash	No	51(96.15%)	32(96.97%)	16(94.11%)	0.717
	Yes	1(3.25%)	1(3.03%)	1(5.88%)	
NS1	Positive	35(67.30%)	22(66.67%)	13(76.47%)	0.767
	Negative	16(30.76%)	11(33.33%)	4(23.53%)	
IgM	Positive	23(44.23%)	16(48.48%)	7(41.18%)	0.883
	Negative	28(53.84%)	17(51.52%)	10(58.82%)	
IgG	Positive	43(82.69%)	27(81.82%)	15(88.24%)	0.840
	Negative	8(15.38%)	6(18.8%)	2(11.76%)	
IgM&IgG Ratio	Primary	28(53.84%)	20(60.61%)	10(58.82%)	0.868
	Secondary	23(44.23%)	13(39.39%)	7(41.18%)	
Comorbidities	Absent	42(80.76%)	26(78.79%)	14(82.35%)	0.951
	Present	10(19.23%)	7(21.21%)	3(17.65%)	
USG findings (n=96 was done)	**Abnormal**	**1(1.92%)**	**19(57.58%)**	**16(94.11%)**	**0.001**
	Normal	**49(94.23%)**	**10(30.30%)**	**1(5.88%)**	
Platelet transfusion	**Received**	**1(1.92%)**	**13(39.39%)**	**9(52.94%)**	**0.001**
	Not received	**51(98.07%)**	**20(60.61%)**	**8(47.06%)**	
Serotype (n=87 was detected)	Denv-2	21(40.38%)	15(45.45%)	6(35.29%)	0.746
	Denv-3	25(48.07%)	13(39.39%)	7(41.18%)	

All the clinical data are represented as N (%). SD-Severe Dengue, DWW-Dengue with warning signs, DWOW- Dengue without warning signs. *Indicates the P ≤ 0.05

Table 3: Biochemical and hematological parameters during Febrile and critical phase of study group

Variables	FEBRILE PHASE				CRITICAL PHASE			
	DWOW	DWW	SD	P-value	DWOW	DWW	SD	P-value
Platelet count (150000-450000)	**41 (78.84%)**	**32 (97.0%)**	**16 (94.11%)**	**0.006**	45 (86.53%)	31 (93.9%)	16 (94.1%)	0.448
	11 (33.33%)	**1 (3.0%)**	**1 (5.88%)**		7 (13.46%)	2 (6.1%)	1 (5.88%)	

All the clinical data are represented as N (%).SD-Severe Dengue; DWW-Dengue with warning signs; DWOW- Dengue without warning signs.

Table 4: Multivariate Analysis for Febrile &Critical Phase of Dengue Infection

Febrile Phase						
Source	**Dependent Variable**	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**P-value**
Intercept	Classification	76.193	1	76.193	146.510	0.001
	Serotype	57.599	1	57.599	243.644	0.001
	IgM & Ig G Ratio	74.361	1	74.361	287.099	0.001
AST DOA	Classification	0.458	1	0.458	0.880	0.352
	Serotype	**2.390**	**1**	**2.390**	**10.111**	**0.002**
	IgM & Ig G Ratio	0.082	1	0.082	0.316	0.576
Critical Phase						
Source	**Dependent Variable**	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**P-value**
Intercept	Classification	93.785	1	93.785	226.493	0.001
	Serotype	65.387	1	65.387	295.528	0.001
	IgM & Ig G Ratio	90.812	1	90.812	345.470	0.001
AST DOD	**Classification**	**1.656**	**1**	**1.656**	**3.999**	**0.050**
	Serotype	0.051	1	0.051	0.231	0.633
	IgM & Ig G Ratio	0.114	1	0.114	0.434	0.513
ALT DOD	**Classification**	**5.668**	**1**	**5.668**	**13.689**	**0.001**
	Serotype	0.022	1	0.022	0.101	0.752
	IgM & Ig G Ratio	0.112	1	0.112	0.426	0.517

Table 5. Binary classification performance of different ML models in dengue severity

Model		Logistic Regression	Naive Bayes	Random Forest	Ridge classifier	Support Vector Machine	XGB
DWOW Vs. DWW	Train accuracy	0.82	**0.89**	1	1	1	0.80
	Test accuracy	0.70	**0.76**	0.70	0.64	0.70	0.70
	Precision	0.81	**0.76**	0.73	0.65	0.71	0.81
	Recall	0.71	**0.76**	0.71	0.65	0.71	0.71
	F1 score	0.67	**0.76**	0.69	0.65	0.70	0.67
	Train AUC	0.76	**0.86**	1	1	1	0.74
	Test AUC	0.68	**0.76**	0.69	0.64	0.70	0.68
DWOW vs. Severe	Train accuracy	1	0.95	1	**1**	1	1
	Test accuracy	0.91	0.86	0.91	**0.86**	0.95	0.86
	Precision	0.92	0.88	0.92	**0.93**	0.96	0.89
	Recall	0.91	0.87	0.91	**0.87**	0.96	0.87
	F1 score	0.90	0.88	0.90	**0.88**	0.95	0.84
	Train AUC	1	0.92	1	**1**	1	1
	Test AUC	0.75	0.82	0.75	**0.92**	0.87	0.62
DWW Vs. Severe	Train accuracy	0.85	**0.74**	0.97	0.85	1	1
	Test Accuracy	0.60	**0.80**	0.80	0.53	0.53	0.66
	Precision	0.60	**0.84**	0.84	0.57	0.57	0.52
	Recall	0.60	**0.80**	0.80	0.53	0.53	0.67
	F1 score	0.60	**0.75**	0.75	0.55	0.55	0.59
	Train AUC	0.82	**0.65**	0.96	0.83	1	1
	Test AUC	0.48	**0.62**	0.62	0.44	0.44	0.45

6.2 Serotyping analysis

Serotyping using type-specific primers revealed DENV2 (n-44) and DENV3 (n-45) serotype among single serotype infections and there is no concurrent infection. This shows that Dengue serotype 2 and Dengue serotype 3 was more prevalent in our sample population collected from 2021 to 2022. The gel image of samples positive for dengue universal and dengue serotypes are depicted in **Fig 1** and **Fig 2 A, B,** respectively. Further, the output of BLAST analysis of sequencing results is represented in **Fig 3.**

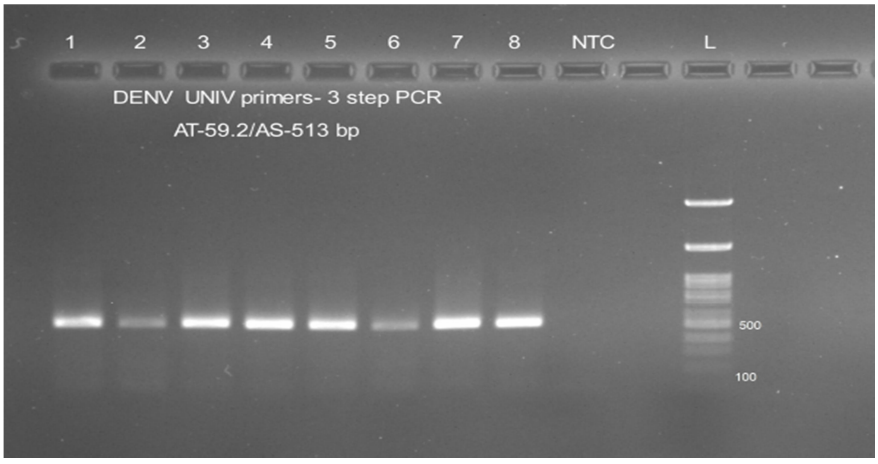

Fig 1. Gel Image of Samples Positive for Dengue Universal

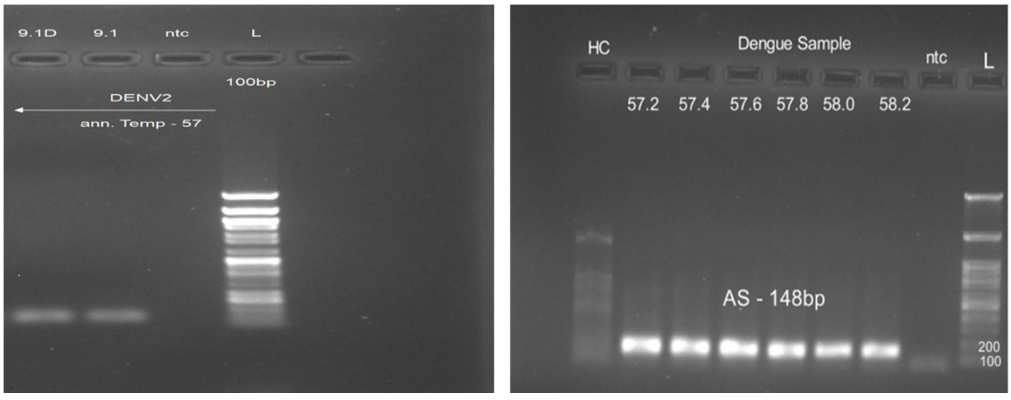

Fig 2. Gel Image of Samples Positive for Dengue Serotype. (A) Gel Image of DENV-2(B) Gel Image of DENV-3

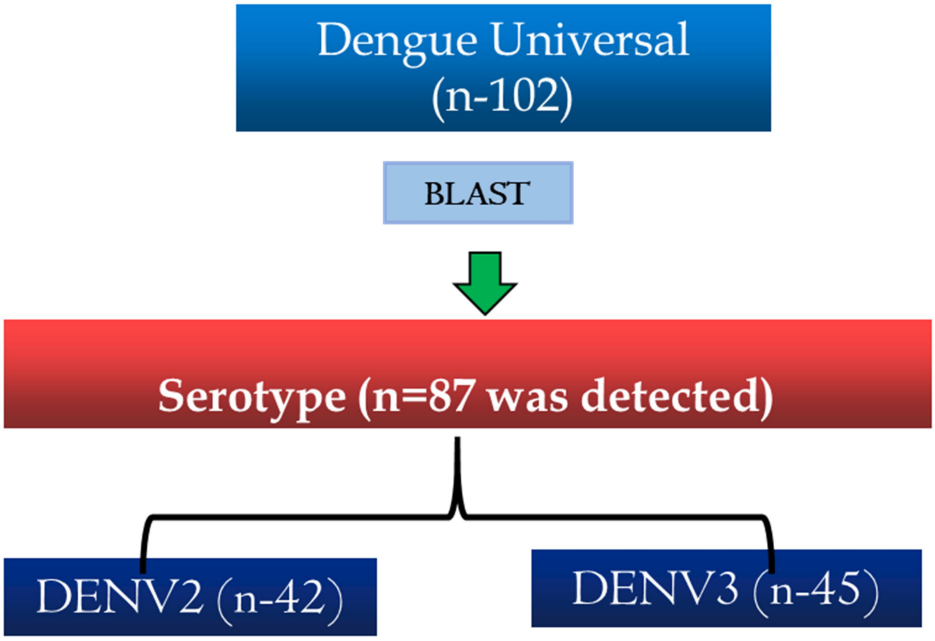

Fig 3. BLAST Results of DENV Serotypes

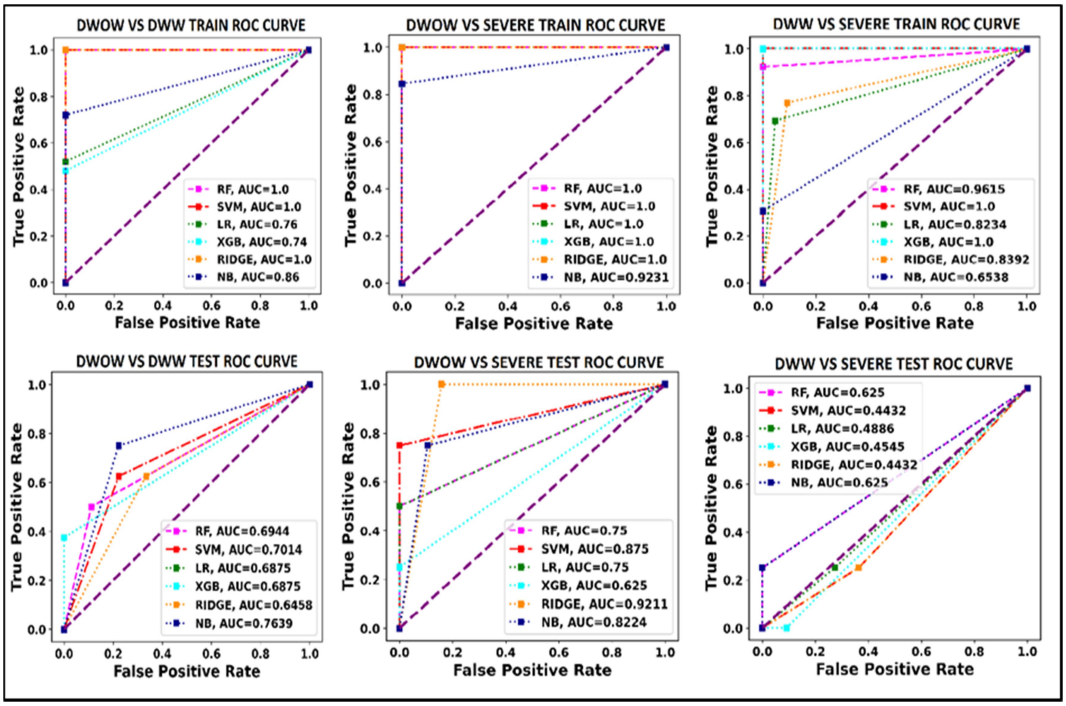

Fig 4. Area Under Curve plot of Dengue severity

6.3 mRNA Levels of Sphingolipid marker and key enzymes

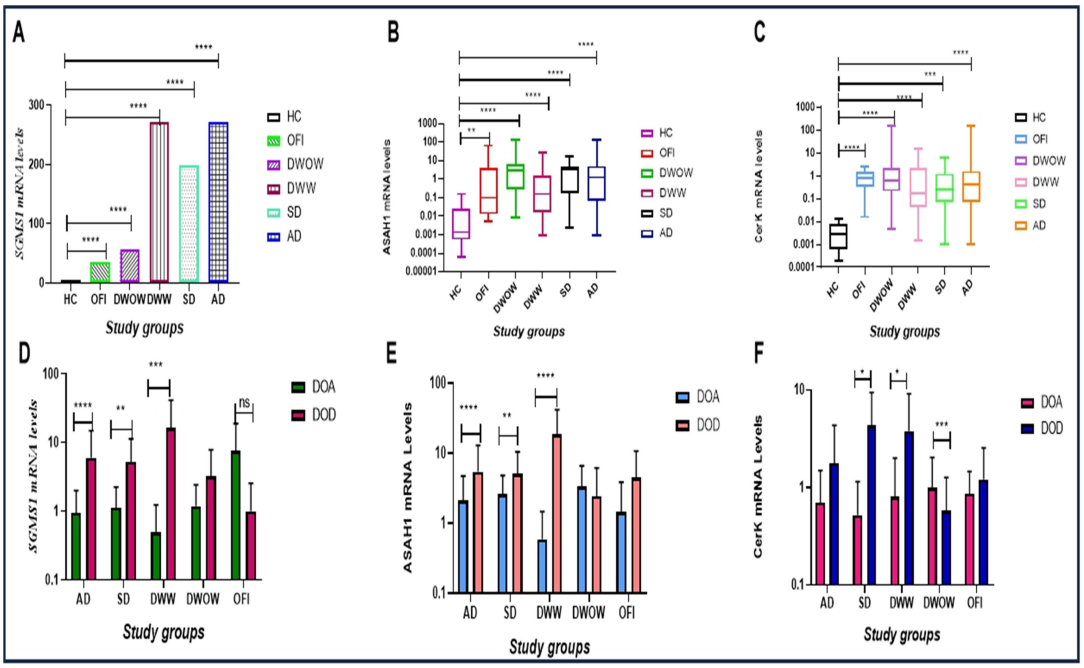

Fig 5. **Expression pattern of SMS1, ASHA1 & CERK transcripts at the time of admission**. Gene expression was measured by qRT-PCR and normalized by β-Actin. Mann-Whitney U test was used to compare the gene expression between different groups. P-value ≤ 0.05 is considered significant.

6.4 Plasma levels of Sphingosine-1-phosphate and Sphingolipid key enzymes using ELISA

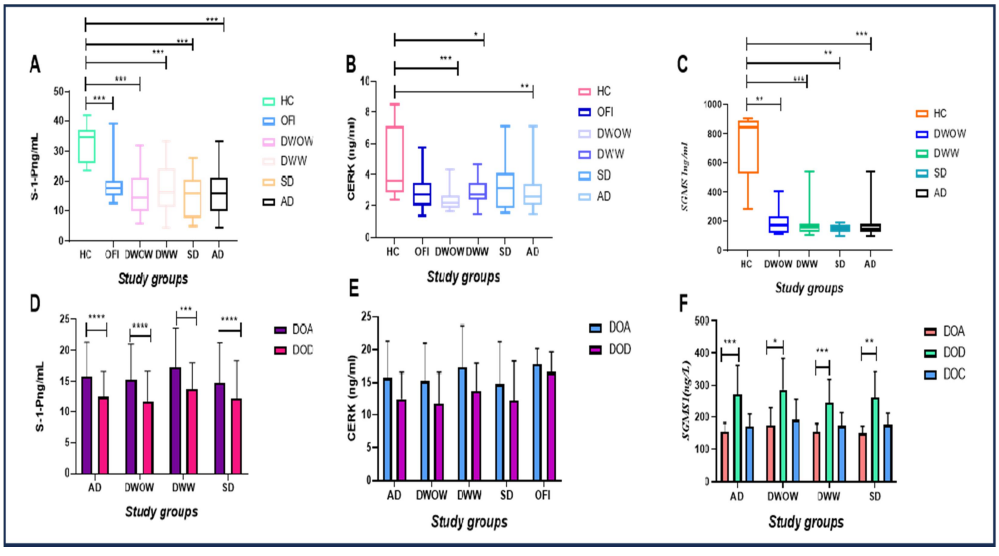

Fig 6. Plasma levels of S1P, CERK & SMS1 at the time of admission. Mann-Whitney U test was used to compare between different groups. P-value ≤ 0.05 is considered significant

Plasma levels of SMS1 from DOA to DOD

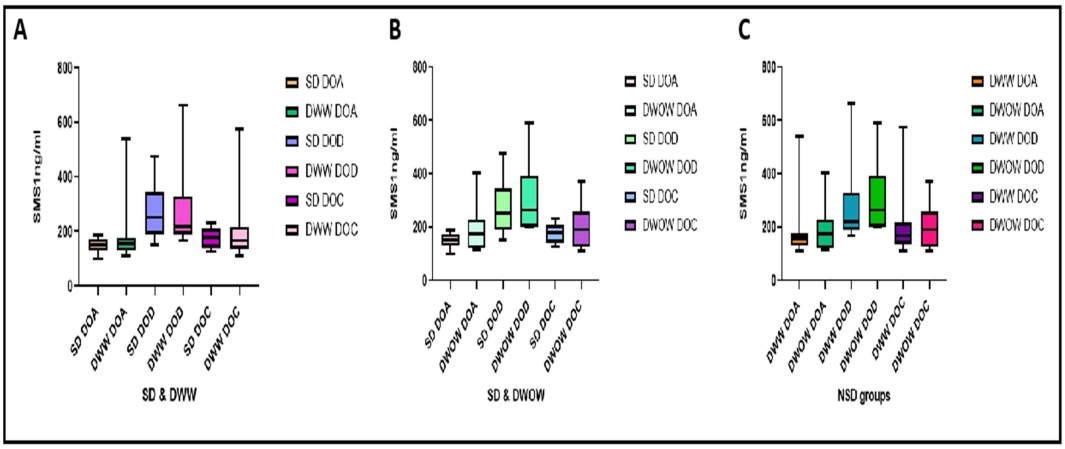

Fig 7. Plasma levels of SMS1 within the study groups. (A) SMS1 levels at SD vs DWW from DOA to DOD. (B) SMS1 levels at SD vs DWOW from DOA to DOD. (C) SMS1 levels at NSD from DOA to DOD. Wilcoxon Signed Rank test was used to compare within groups, respectively. Results are expressed in the Median (IQR).

Plasma levels of S1P from DOA to DOD

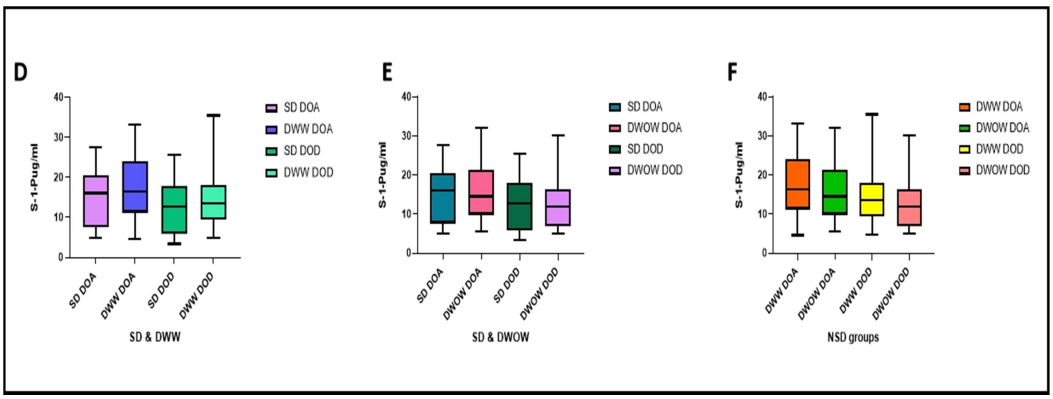

Fig 8. Plasma levels of S1P within the study groups (D) S1P levels at SD vs DWW from DOA to DOD. (E) S1P levels at SD vs DWOW from DOA to DOD. (F) S1P levels at NSD at DOA to DOD. Wilcoxon Signed Rank test was used to compare within groups, respectively. Results are expressed in the Median (IQR).

Plasma levels of CERK from DOA to DOD

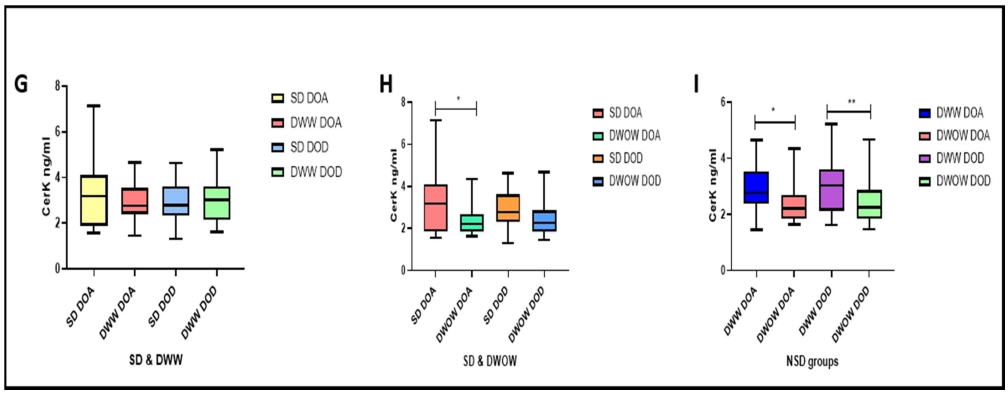

Fig 9. Plasma levels of CERK within the study groups. (G) CERK levels at SD vs DWOW from DOA& DOD(H) CERK levels at SD vs DWW from DOA to DOD(I)CERK levels at NSD from DOA to DOD. Wilcoxon Signed Rank test was used to compare within groups, respectively. Results are expressed in the Median (IQR).

In Vitro Analysis: Levels of S1P in activated ECs

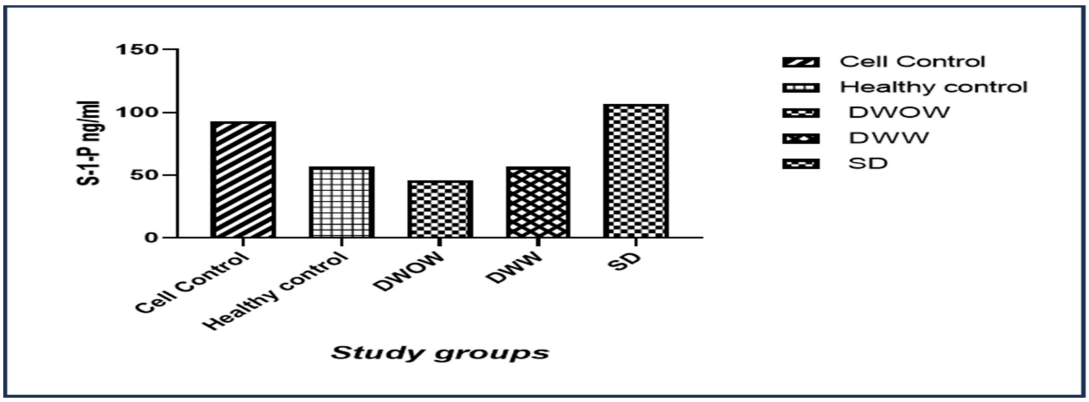

Fig 10. Level of S1P in HUVEC cells treated with Dengue, and Control Serum (HC & FBS). HC-Healthy Control; DWOW - Dengue without Warning Sign; DWW-Dengue with Warning Sign, SD – Severe Dengue.

6.4.4 Different machine learning models for predicting disease severity

AUC Curve Interpretation for S1P & CERK along with clinical parameters during Febrile Phase

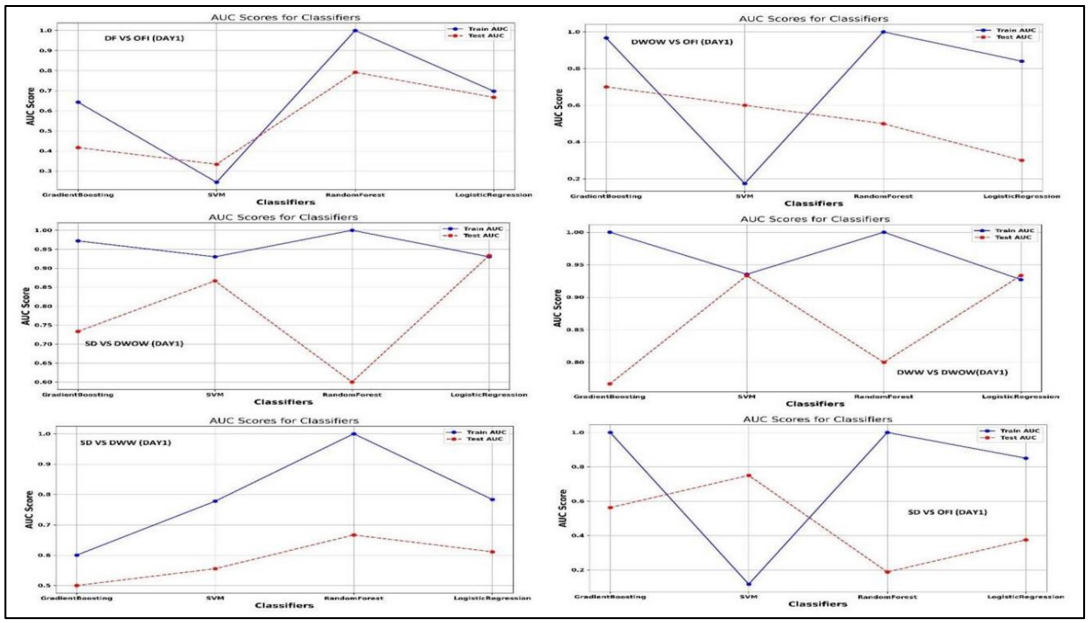

Fig 11: AUC plot showed train and test scores of all classifiers along with groups at DOA

AUC Curve comparing S1P & CERK along with clinical parameters during Critical Phase

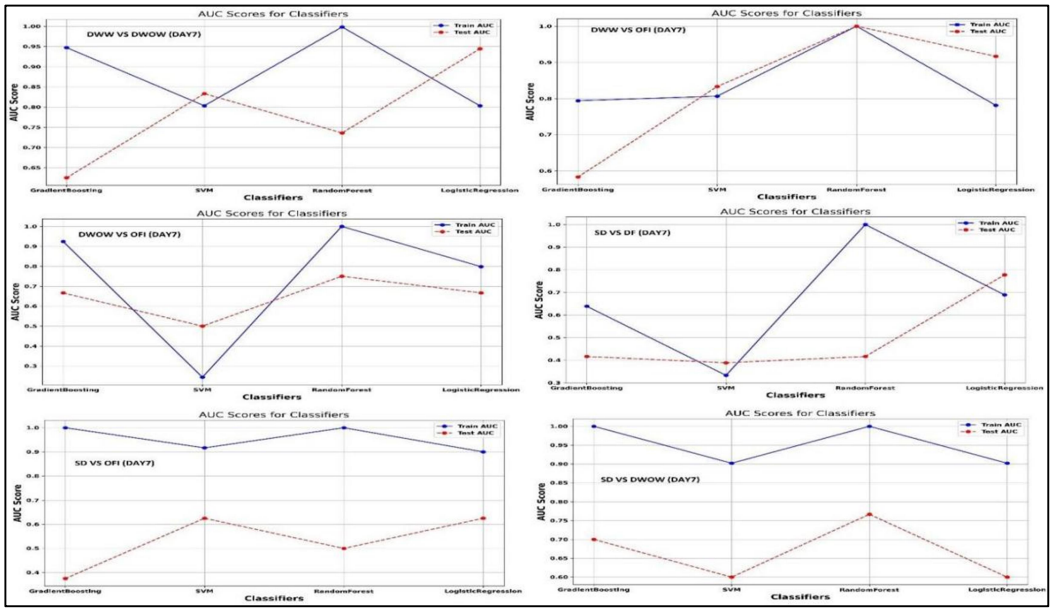

Fig 12: AUC plot showed train and test scores of all Classifiers along with groups during DOD

6.4.4 Association between Sphingolipid molecules and disease severity markers

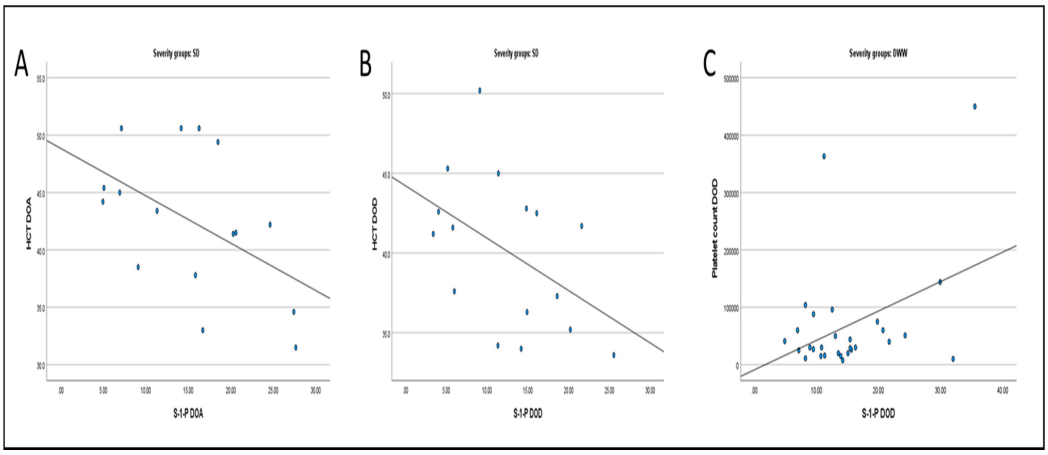

Fig14. Correlation between clinical parameters and sphingolipid-related enzyme molecules during dengue infection.(A) Negative correlation between HCT and S1P levels of SD cases at DOA (R = - 0.503; P ≤ 0.047);(B) Negative correlation between HCT and S1P levels of SD cases at DOD (R = 0.742; P ≤ 0.014); (C) Positive correlation between platelet count and S1P levels of DWW cases at DOD (R = 0.391; P ≤ 0.03).Since the data was non-parametric, Spearman's Rho Correlation was used to compare the data. P-value ≤ 0.05 is considered significant.

C18:1 Ceramide Vs Severity markers in DWW cases

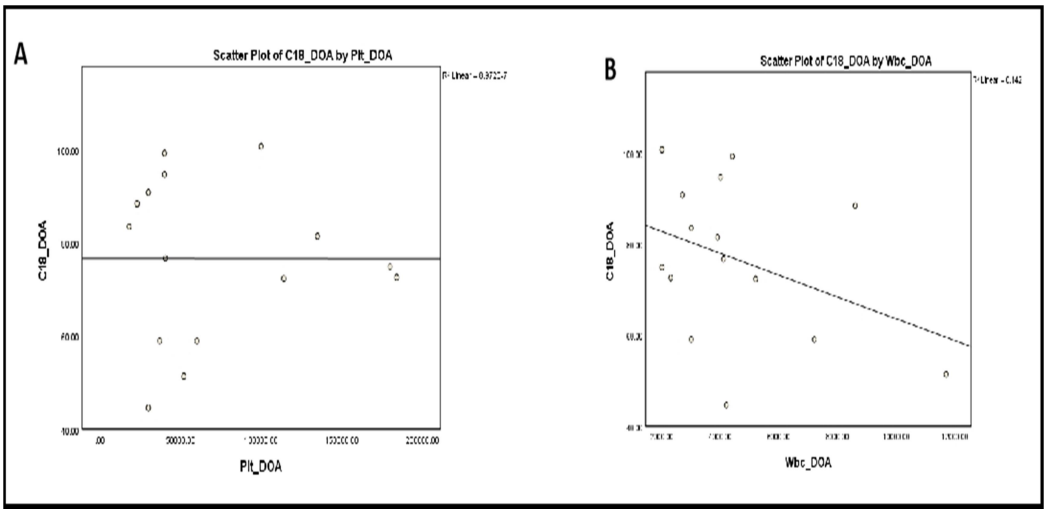

Fig 17. Negative correlations between clinical parameters and C18:1 during dengue infection. (A) Correlation between Platelet count & C18: 1 levels of DWS+ cases at DOA (R = -0.161; P ≤ 0.01); (B) Correlation between WBC and C 18:1 levels of DWS+ cases at DOA (R = -0.585; P ≤ 0.02)

6.4.6 Quantification of C 18:1 Ceramide using HPLC Analysis

HPLC Chromatograms of C18:1 Ceramide in the study groups

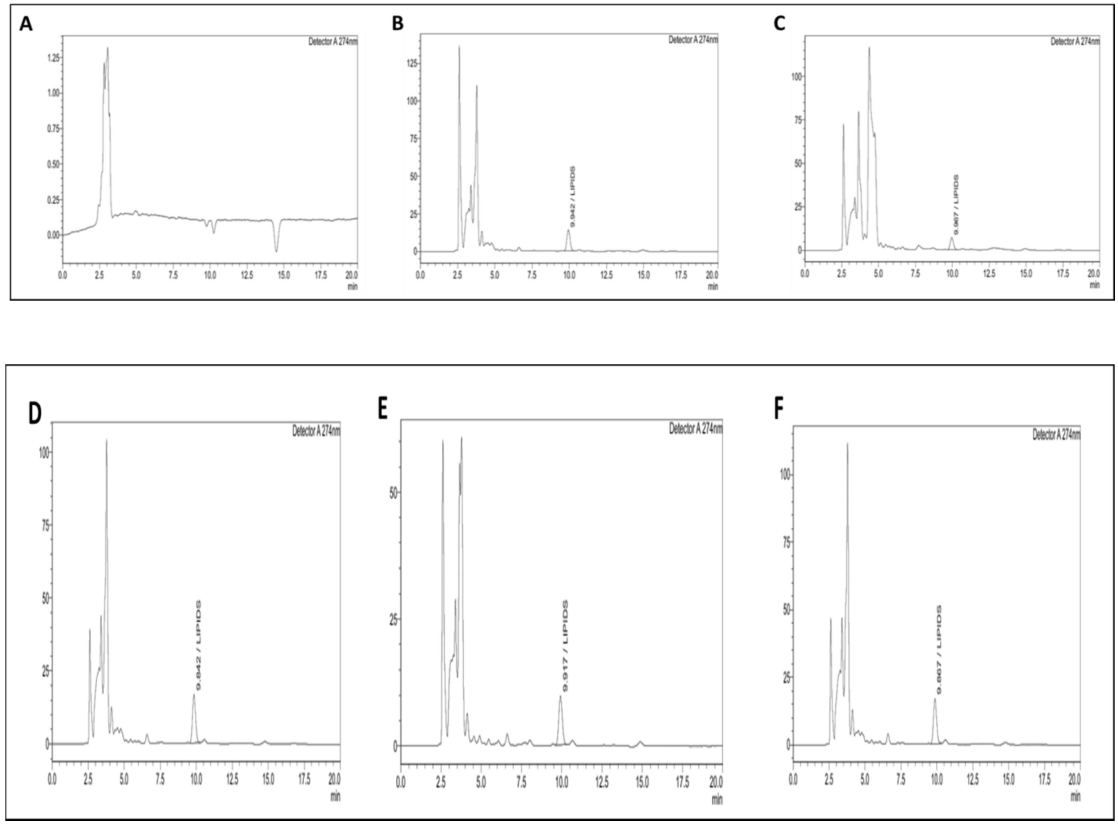

Fig18. Chromatogram view of C18:1 Ceramide levels (µg/mL) in Dengue and Controls (HC & OFI). (A) Blank of C18:1 Ceramide; (B) Healthy Control of C18:1 Ceramide; (C) OFI of C18:1 Ceramide; (D) SD of C18:1 Ceramide ;(E) DWS+ of C18:1 Ceramide (F) DWS- of C18:1 Ceramide

Plasma Levels of C18:1 Ceramide in study groups

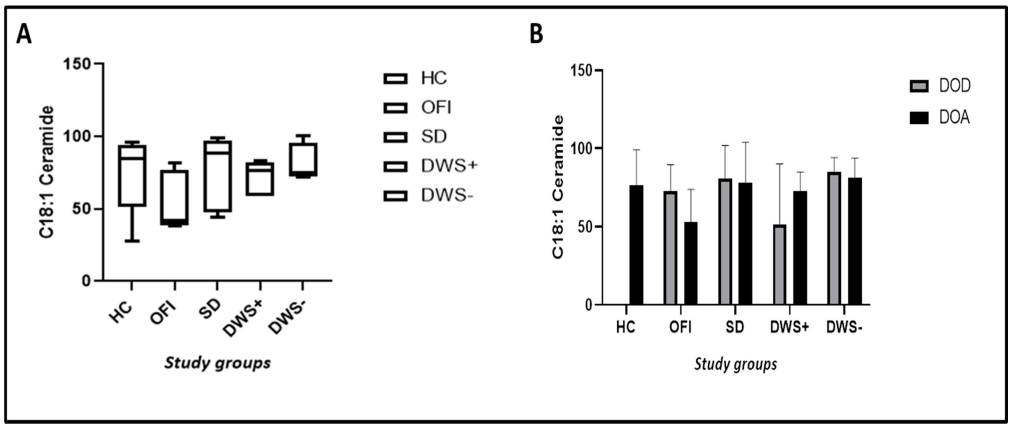

Fig 19. Levels of C18:1 Ceramide (µg/mL) in Dengue, OFI, and Health Control at the time of Admission. (A) Levels of C18:1 Ceramide in Study groups; (B) Levels of C18:1 Ceramide levels during phase of infection

Plasma Levels of C18:1 Ceramide in between the groups

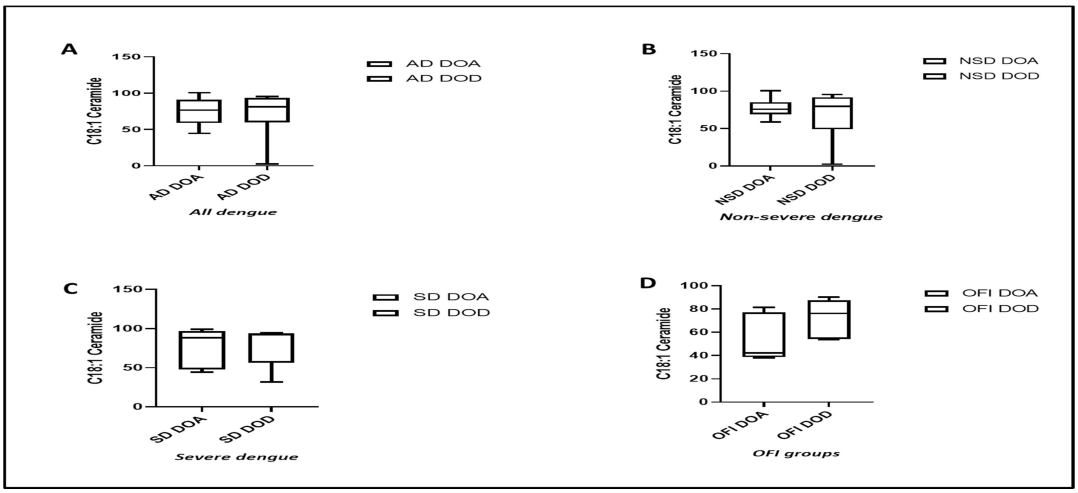

Fig 20. Expression of C18:1 Ceramide levels (µg/mL) in Dengue and OFI (A) Levels of C18:1 Ceramide in AD cases; (B) Levels of C18:1 Ceramide levels in NSD cases; (C) Levels of C18:1 Ceramide in SD cases; (D) Levels of C18:1 Ceramide in OFI cases.

7. DISCUSSIONS

The study aimed to identify early prognostic indicators for severe dengue based on clinical characteristics and standard laboratory examinations across different clinical forms of dengue. The analysis of 102 dengue patient samples collected between 2021-2022 revealed males (62.74%) were more affected than the females (37.25%), which is supported with the another study(33).In the present study confirmed the prevalence of DENV2 and DENV3 serotypes, which is in line with earlier reports (34–36).The results were supported with previous study by Lakra et al., 2023 showed that the nausea (58.8%) and bleeding (52.9%) were highly correlated with SD (OR: 0.030, 95% CI: 0.004-0.246)(37). Bleeding (82.6%) was linked to severe thrombocytopenia (median platelet count: 10,000, Increased hematocrit (>20%) is an early sign of plasma leakage. However, no strong correlation was found between hematocrit and severity, consistent with previous studies(38).A combination of nausea, bleeding, platelet transfusion, AST, and USG findings can help differentiate dengue fever from severe dengue.ML-based prediction system identified key risk factors for dengue severity, including IgM/IgG ratio, abdominal pain, bleeding symptoms, PCV, hemoglobin, chloride, gender, neutrophils, lymphocytes, creatinine, AST, ALT, rash, and dry cough our results supports the previous study by Tiruveedhula et al. 2018(39).

Circulating molecules, particularly those released by lipids, platelets, ECs, and monocytes/macrophages, are thought to be the crucial mediators in thrombocytopenia and vascular leakage as observed in severe forms of dengue. So, the current study evaluated the transcriptional expression profiles of selected molecules associated with sphingolipid (S1P, Ceramide) and their key enzymes (SMS1, ASHA1, & CERK) at the three distinct clinical stages of dengue compared to

OFI and healthy individuals. Plasma Sphingosine-1-phosphate (S1P) levels significantly dropped during the defervescence phase of dengue, especially in severe cases ($P < 0.01$).Compared to healthy controls there showed a lowered serum S1P levels in dengue subjects in consistent to earlier report by Goms et al., 2014(40).SD patients showed low S1P levels, increased HCT, and a significant negative correlation between S1P and HCT during the febrile and critical phases. Similarly, low S1P correlated positively with low platelet counts. These findings align with (Michels M et al. 2015), suggesting S1P influences plasma leakage independently(41).SMGS1 activity variations may alter sphingomyelin and ceramide levels, affecting S1P production and endothelial integrity. CERK phosphorylates ceramide to C1P, balancing its protective and pro-apoptotic effects. In dengue, such imbalances can worsen vascular permeability, with elevated C1P contributing to severe complications(23,38). In vitro and in vivo studies revealed that knocking down SMGS1 reduced the adhesion, entry, and replication of influenza, Japanese encephalitis, and rubella viruses(42,43). Additionally, SMGS1-deficient cells showed lower expression of influenza viral proteins on their surface and impaired viral entry, suggesting a potential cellular defense mechanism against enveloped viruses(44).

According to the study result, the levels of both mRNA and protein levels of CERK are lower than those of healthy individuals. Previous study reported that the host's cellular machinery may have assisted in lowering ceramide kinase levels during an active dengue illness to manage apoptosis and other adverse effects brought on by an increase in ceramides(23).Similarly, sphingomyelin synthase 1(SMS1), another enzyme, aids in the breakdown of ceramide and the production of sphingomyelin, which is a precursor to sphingosine-1-phosphate reported by Yagi et al 2023 (43).To strengthen our findings, we used a machine learning approach to assess the level of

S1P, sphingolipid enzymes, platelets, HCT, albumin, AST/ALT, and other parameters to predict the disease severity. SVM performed good and predicted that CERK enzyme, along with platelet count, HCT, and ALT could be useful markers to predict the severity of the diseases compared with DWOW. All these cellular processes may ultimately result in plasma leakage and thrombocytopenia. However, further in vitro studies are necessary to elucidate the role of our selected sphingolipid and key enzyme markers in the progression of dengue disease. Additionally, a large-scale cohort study in a clinical setting may be needed to confirm the significance of these markers in the early prognosis of dengue disease outcomes.

8. SUMMARY OF THE RESULTS

1. Males were more associated with severe dengue (SD) than females, and younger individuals (18-27 years) were more prone to SD.
2. DENV-2 and DENV-3 were the most prevalent serotypes during 2021-2022.
3. Abnormal ultrasound findings (OR=0.011), platelet transfusion (OR=0.030), and low lymphocyte levels (OR=0.88) were significantly associated with SD ($p<0.05$).
4. Low creatinine levels (OR=0.116), high sodium levels (OR=20.51), and elevated AST (OR=3.030) and ALT (OR=0.018) were significantly associated with SD ($p<0.05$).
5. Hematocrit (HCT), AST/ALT ratio, and hemoglobin were pivotal in differentiating SD from non-severe cases.
6. SMS1, ASHA1, and CERK mRNA levels were significantly elevated in both dengue and other febrile illness (OFI) patients compared to healthy controls (HC).
7. SMS1, ASHA1, and CERK mRNA levels were elevated in dengue patients during the critical phase but showed no significant changes in OFI during the day of disease onset (DOD).
8. Dengue patients had significantly lower S1P, CERK, and SMS1 levels in plasma compared to HC ($p<0.001$).
9. Plasma S1P levels declined markedly with disease progression in all dengue types (AD, DWOW, DWW, and SD) and in OFI.
10. When HUVEC cells were exposed to dengue patient serum, S1P levels were higher in SD cases compared to untreated cells and serum from DWOW and HC.
11. SVM Model Prediction: Support Vector Machine (SVM) predicted that ceramide kinase enzyme, platelet count, HCT, and ALT could be useful severity markers compared to DWOW.

12. S1P, combined with HCT, platelet count, AST, and albumin, could enhance severity prediction.
13. Ceramide levels were significantly elevated in dengue patients, with the highest levels in SD cases, particularly during the critical phase.
14. Machine learning models highlighted the importance of ceramide-related pathways in differentiating SD from non-severe cases.
15. The study suggests that a combination of molecular and clinical markers could improve the early prediction and management of severe dengue cases.

CONCLUSION

- From the study it can be inferred that clinical signs such as bleeding and nausea, biochemical parameters like liver enzymes and platelet counts, and radiological findings of USG were linked with disease severity.
- Study showed a significant reduction in S1P levels across all dengue forms during febrile phases, with further decline in SD during the critical phase ($P < 0.05$) mRNA levels of the enzymes were increasing during critical phase ($P \leq 0.001$) with no significant difference noted in their respective protein levels.
- S1P and SGMS1 levels correlated significantly with clinical severity indicators, including hematocrit, albumin, platelet count, and liver enzymes.
- SVM analysis identified CERK levels along with platelet count, HCT, and ALT as markers with high predictive accuracy for dengue severity.
- During defervescence, ceramide levels increased in dengue patients, with higher levels in severe dengue (SD) compared to non-severe dengue (DWS+ & DWS-) and OFI, particularly during the critical phase.
- To the best of our knowledge this is the first study that demonstrated an association with sphingolipids and its key enzymes in the pathogenesis of dengue.

9. LIMITATION OF THE STUDY

1. The small number of severe cases enrolled to classify dengue infections. A larger sample is needed to confirm the roles of S1P and its related enzymes (ASAH1, CERK, and SGMS1).
2. This study focused only on adults, so the results may not apply to pediatric dengue patients.
3. Understanding sphingolipid markers and key enzymes in dengue pathogenesis could aid biomarker identification and therapeutic development.

FUTURE PERSPECTIVES

1. USG testing at an earlier phase of dengue and a point-of-care system for the quantification of AST/ALT levels may lead to an early prediction of SD.
2. Understanding of the molecular mechanisms underlying dengue pathogenesis.
3. The present studies shed light on disease severity, offer potential diagnostic markers, and suggest avenues for therapeutic intervention.

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WORK PRESENTED IN CONFERENCES

- Poster presentation on "National Level Conference on Biotechnology and OMICS Sciences-2022 organized by Department of Biotechnology, Alagappa University" on May 27, 2022.
- Poster presentation on "12th Annual Conference of Clinical Infectious Disease Society organized by CIDSCON 2022" on July 15th to 17th, 2022.
- Oral presentation at 16th International Conference of Medical Arthropodology (ICMA 2023) on "Emerging and Re-emerging Infectious Vector-borne & Zoonotic Diseases: Impact of Climate Change and Global Warming" on December 13th to 15th, 2023.
- Oral presentation entitled "Analysis of total ceramides by RP-HPLC and its association with hematocrit in dengue patients." in SBV Research Days on 25th Feb 2021-2022,Organized by Deanery of Research, Sri Balaji Vidyapeeth, Puducherry.